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Abstract: Background We investigated the feasibility of rapid disc diffusion antibiotic susceptibility testing (rAST) with reading of inhibition zones after 6 and/or 8 h of incubation for *Enterococcus faecalis*, *Enterococcus faecium*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. In addition, we evaluated discrimination of resistant populations from the WT populations at early timepoints and the requirement for clinical breakpoint adaptations for proper interpretation of rAST data. Methods In total, 815 clinical strains [*E. faecalis* (n = 135), *E. faecium* (n = 227), *P. aeruginosa* (n = 295) and *A. baumannii* (n = 158)] were included in this study. Disc diffusion plates were streaked, incubated and imaged using the WASPLabTM automation system. WT populations and non-WT populations were defined using epidemiological cut-offs. Results and conclusions rAST at 6 and 8 h was possible for *A. baumannii* and enterococci with readability of inhibition zones >90%. Overall categorical agreement of rAST at 6 h with AST at 18 h was 97.2%, 97.4% and 95.3% for *E. faecalis*, *E. faecium* and *A. baumannii*, respectively. With few exceptions, major categorization error rates were <1% for *A. baumannii*, and vancomycin-resistant *E. faecium* were clearly separated from the WT at 6 h. For *P. aeruginosa* the average readability of inhibition zones was 68.9% at 8 h and we found an overall categorical agreement of 94.8%. Adaptations of clinical breakpoints and/or introduction of technical buffer zones, preferably based on aggregated population data from various epidemiological settings, are required for proper interpretation of rAST.

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Rapid disc diffusion antibiotic susceptibility testing for *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Enterococcus* spp.

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Background: We investigated the feasibility of rapid disc diffusion antibiotic susceptibility testing (rAST) with reading of inhibition zones after 6 and/or 8 h of incubation for *Enterococcus faecalis*, *Enterococcus faecium*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. In addition, we evaluated discrimination of resistant populations from the WT populations at early timepoints and the requirement for clinical breakpoint adaptations for proper interpretation of rAST data.

Methods: In total, 815 clinical strains [*E. faecalis* ($n = 135$), *E. faecium* ($n = 227$), *P. aeruginosa* ($n = 295$) and *A. baumannii* ($n = 158$)] were included in this study. Disc diffusion plates were streaked, incubated and imaged using the WASPLab™ automation system. WT populations and non-WT populations were defined using epidemiological cut-offs.

Results and conclusions: rAST at 6 and 8 h was possible for *A. baumannii* and enterococci with readability of inhibition zones >90%. Overall categorical agreement of rAST at 6 h with AST at 18 h was 97.2%, 97.4% and 95.3% for *E. faecalis*, *E. faecium* and *A. baumannii*, respectively. With few exceptions, major categorization error rates were <1% for *A. baumannii*, and vancomycin-resistant *E. faecium* were clearly separated from the WT at 6 h. For *P. aeruginosa* the average readability of inhibition zones was 68.9% at 8 h and we found an overall categorical agreement of 94.8%. Adaptations of clinical breakpoints and/or introduction of technical buffer zones, preferably based on aggregated population data from various epidemiological settings, are required for proper interpretation of rAST.

Introduction

Owing to increasing antibiotic resistance, drug susceptibility patterns of bacterial pathogens become more and more unpredictable and empirical first-line therapy often turns out to be inadequate.^{1,2} Rapid disc diffusion antibiotic susceptibility testing (rAST) would be beneficial for adequate patient care and proper installation of targeted antimicrobial therapy, significantly improving clinical outcome and reducing mortality.^{3–6}

Automated antimicrobial susceptibility test devices such as VITEK-II or Phoenix™ in principle are able to deliver test results within 8–16 h, but for some species/drug combinations significant error rates have been demonstrated, e.g. for *Pseudomonas aeruginosa* and β -lactams such as cefepime, ceftazidime, carbapenems and piperacillin/tazobactam.^{7,8} Another weakness of automated microdilution is a poor sensitivity for important resistance

phenotypes such as VRE or carbapenem-resistant *Acinetobacter baumannii*.^{9,10}

Molecular detection of resistance determinants delivers rapid results but is hampered by the high number of genes to be covered for adequate sensitivity and by the lack of validated phenotype/genotype knowledge databases, particularly for *P. aeruginosa* and enterococci.^{11,12} Maintenance of an accurate coverage is a complex task taking into account the geographical differences in the prevalence of resistance-mediating genes. In addition, the mere presence of a gene encoding for antibiotic resistance does not necessarily correlate with its phenotypic expression.¹¹

Disc diffusion is an established, accurate and standardized procedure, which can be adapted to a diagnostic laboratory's needs. EUCAST and CLSI recommend an incubation time of 16–18 h for most species/drug combinations.^{13,14} In a proof-of-principle study, we recently demonstrated that automated rAST is feasible.¹⁵ We

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here evaluated whether early reading of disc diffusion is possible for clinical pathogens with a high diversity of intrinsic resistance mechanisms, namely enterococci, *P. aeruginosa* and *A. baumannii*. The aims of this study were to examine the feasibility of rAST to discriminate important resistance phenotypes from WT populations and to identify species/drug combinations that need adaptation of clinical breakpoints (CBPs).

Methods

Clinical isolates

Study isolates were selected covering a range of inhibition zone diameters from 6 to 40 mm for each species/drug combination tested (Figure S1, available as [Supplementary data](#) at JAC Online). In particular, isolates close to the CBPs were included. All non-duplicate clinical strains included in this study were isolated over a 3 year period from 2013 until 2016 in the clinical microbiology laboratory of the Institute of Medical Microbiology, University of Zurich. Isolates of the same species were considered duplicate(s) if they (i) originated from the same patient, and (ii) showed one major and two minor differences in AST interpretation at maximum. The following numbers of clinical isolates were tested: *Enterococcus faecalis* ($n = 135$), *Enterococcus faecium* ($n = 227$), *P. aeruginosa* ($n = 295$) and *A. baumannii* ($n = 158$).

Quality control strains

To control for methodological precision and for calibration to EUCAST CBPs, *E. faecalis* ATCC 29212 and *P. aeruginosa* ATCC 27853 EUCAST quality control (QC) strains were tested daily from individual fresh subcultures and individually prepared 0.5 McFarland standards. Interpretation was done according to EUCAST QC tables version 6.1.¹⁶ QC ranges and targets were fulfilled during this study (data not shown).

Definition of phenotypes

Resistance phenotypes and the WT populations were defined using EUCAST epidemiological cut-offs (ECOFFs; Table S1).¹⁷ Prior to conducting this study drug susceptibility was assigned on the basis of independent disc diameter measurements applying the EUCAST recommended method on Mueller-Hinton II agar (Beckon-Dickinson, Franklin Lakes, NJ, USA) using antibiotic discs from i2a (Montpellier, France) and automated recording using the SirSCAN™/SirWeb™ system (i2a).

We screened for vancomycin resistance in enterococci according to the EUCAST guidelines using a 24 h incubation period.¹⁸ Briefly, isolates of *E. faecalis* and *E. faecium* with vancomycin inhibition zone diameters ≥ 12 mm and sharp zone edges were considered vancomycin susceptible, i.e. WT. Isolates with vancomycin inhibition zones < 12 mm, isolates with fuzzy zone edges (regardless of the inhibition zone diameter) or isolates with colonies within the inhibition zone were subjected to PCR assays targeting *vanA* and *vanB* to confirm the vancomycin-resistant phenotype.¹⁹ For clinical isolates of *E. faecalis* with an ampicillin non-WT phenotype, identification was reconfirmed from the AST plate.

Automated susceptibility testing

Susceptibility testing was performed as described previously according to EUCAST guidelines version 6.0, which are essentially the same as that of CLSI 2016 for the organisms of this study.^{13–15} In brief, bacterial suspensions were manually adjusted to a turbidity equivalent to that of a 0.5 McFarland standard and processed within 15 min. Mueller-Hinton II agar plates (Oxoid Limited, Basingstoke, UK) were processed in the fully automated WASP™ (Copan Italia, S.p.A., Brescia, Italy), i.e. plates were each inoculated with 60 μ L of the bacterial suspension and streaked automatically. Antibiotic discs of a

single production lot (Oxoid Limited) were placed using a standard distributor, which was handled by a WASP™ AST robot immediately after plate streaking. Subsequently, plates were automatically transported to and incubated in a WASPLab™ incubator (Copan) at 36 ± 2 °C in ambient air. Images were taken after 6, 8, 12 and 18 h of incubation under continuous temperature conditions. Diameter measurements were automatically done by the WASPLab™ reading software (Copan) and were, if necessary, adjusted on-screen by an experienced technician.

Statistical analyses

All statistical analyses were performed using R, version 3.2.3.²⁰ The R package pROC, version 1.8, was used to calculate areas under the receiver operating characteristic curve and associated confidence intervals.²¹

To quantify the separability of the WT and non-WT populations, the maximal accuracy (i.e. the maximal fraction of true predictions) achievable by a cut-off was calculated based on the prevalences in our dataset.

Results

Readability

Readability was defined as the percentage of data points for which a diameter measurement could reliably be determined for a given species/drug combination.

Enterococci

For *E. faecalis* and *E. faecium* the average readability was $>90\%$ at 6 h, $>95\%$ at 8 h and $>99\%$ at 12 h (Table 1).

Non-fermenters

Average readability for *A. baumannii* was 99.2% at 6 h and 100% at 8 and 12 h. For *P. aeruginosa* inhibition zone diameters were not readable reliably at 6 h, but were readable for 68.9% of all isolates at 8 h and for 93.4% of all isolates at 12 h (Table 1).

Categorical agreement and interpretation errors

Compared with 12 h, categorical agreement at 6–8 h with interpretation at 18 h varied for different species/drug combinations.

Enterococci

Average agreement for *E. faecalis* and *E. faecium* was 97.2% and 97.4% at 6 h and 97.6% and 95.4% at 8 h (Table 1). For *E. faecalis* categorical agreement at 6 h ranged from 91.3% for gentamicin to 100% for vancomycin, and for *E. faecium* categorical agreement at 6 h ranged from 93.1% for gentamicin to 100% for norfloxacin (Table 1). The highest interpretation error rates in both enterococcal species were observed for gentamicin [very major error (vME) rates of up to 8.7%].

Non-fermenters

For *P. aeruginosa* categorical agreement was 94.8% at 8 h and 97.3% at 12 h (Table 1). Average categorical agreement for *A. baumannii* was 95.3% at 6 h and 99.9% at 8 h. For *P. aeruginosa* categorical agreement at 8 h ranged from 89.2% for meropenem to 99% for tobramycin and gentamicin, and for *A. baumannii* categorical agreement at 6 h ranged from 87.8% for amikacin to 99.4% for gentamicin (Table 1). Except for meropenem, categorical agreement

Table 1. Readability and categorical agreement of early zone reading after 6, 8 and 12 h as compared with standard incubation at 18–24 h

	Zone diameter measurements and related classification parameters (all values in %)														
	6 versus 18 h					8 versus 18 h					12 versus 18 h				
	readability	categorical agreement	vME	ME	mE	readability	categorical agreement	vME	ME	mE	readability	categorical agreement	vME	ME	mE
<i>E. faecalis</i> , n = 135															
ampicillin	92.6	99.2	0.0	0.0	0.8	97.8	98.5	0.0	0.0	1.5	99.3	98.5	0.0	0.0	1.5
gentamicin	93.3	91.3	8.7	0.0	0.0	97.8	93.2	6.8	0.0	0.0	99.3	100.0	0.0	0.0	0.0
norfloxacin	94.1	98.4	1.6	0.0	0.0	98.5	98.5	1.5	0.0	0.0	100.0	100.0	0.0	0.0	0.0
vancomycin	93.3	100.0	0.0	0.0	0.0	97.8	100.0	0.0	0.0	0.0	99.3	100.0	0.0	0.0	0.0
average ^b	93.3	97.2	2.6	0.0	0.2	98.0	97.6	2.1	0.0	0.4	99.5	99.6	0.0	0.0	0.5
<i>E. faecium</i> , n = 227															
ampicillin	100.0	99.1	0.0	0.0	0.9	100.0	99.1	0.0	0.0	0.9	100.0	99.1	0.0	0.0	0.9
gentamicin	83.3	93.1	6.9	0.0	0.0	98.7	85.7	14.3	0.0	0.0	100.0	98.2	1.8	0.0	0.0
norfloxacin	93.4	100.0	0.0	0.0	0.0	99.6	99.1	0.9	0.0	0.0	100.0	100.0	0.0	0.0	0.0
vancomycin	89.4	97.5	2.5	0.0	0.0	98.2	97.8	2.2	0.0	0.0	100.0	99.1	0.9	0.0	0.0
average	91.5	97.4	2.4	0.0	0.2	99.1	95.4	4.3	0.0	0.2	100.0	99.1	0.7	0.0	0.2
<i>P. aeruginosa</i> , n = 295 ^a															
piperacillin/tazobactam						68.5	95.5	2.5	2.0	0.0	92.9	96.7	3.3	0.0	0.0
cefepime						68.5	95.5	2.5	2.0	0.0	93.6	97.5	2.5	0.0	0.0
ceftazidime						68.8	95.1	2.5	2.5	0.0	93.2	95.6	3.6	0.7	0.0
imipenem						67.5	93.5	1.5	0.0	5.0	93.2	97.1	0.7	0.0	2.2
meropenem						69.2	89.2	0.0	0.0	10.8	92.9	95.6	0.4	0.0	4.0
gentamicin						69.8	99.0	0.5	0.5	0.0	93.6	99.3	0.7	0.0	0.0
tobramycin						69.8	99.0	1.0	0.0	0.0	93.6	98.9	1.1	0.0	0.0
amikacin						67.8	96.5	0.0	0.0	3.5	93.6	98.2	0.0	0.0	1.8
ciprofloxacin						69.8	90.3	0.0	9.7	0.0	93.6	96.7	0.0	3.3	0.0
average						68.9	94.8	1.2	1.9	2.1	93.4	97.3	1.4	0.4	0.9
<i>A. baumannii</i> , n = 158															
imipenem	99.4	98.7	0.6	0.0	0.6	100.0	99.4	0.0	0.0	0.6	100.0	99.4	0.0	0.0	0.6
meropenem	99.4	91.7	0.6	0.0	7.6	100.0	100.0	0.0	0.0	0.0	100.0	99.4	0.0	0.0	0.6
gentamicin	99.4	99.4	0.0	0.6	0.0	100.0	100.0	0.0	0.0	0.0	100.0	100.0	0.0	0.0	0.0
tobramycin	99.4	96.8	0.0	3.2	0.0	100.0	100.0	0.0	0.0	0.0	100.0	100.0	0.0	0.0	0.0
amikacin	98.7	87.8	0.0	0.6	11.5	100.0	100.0	0.0	0.0	0.0	100.0	100.0	0.0	0.0	0.0
ciprofloxacin	98.7	98.1	0.0	1.9	0.0	100.0	100.0	0.0	0.0	0.0	100.0	100.0	0.0	0.0	0.0
levofloxacin	99.4	94.9	0.0	0.6	4.5	100.0	100.0	0.0	0.0	0.0	100.0	100.0	0.0	0.0	0.0
average	99.2	95.3	0.2	1.0	3.5	100.0	99.9	0.0	0.0	0.1	100.0	99.8	0.0	0.0	0.2

mE, minor error.

Readability was defined as the percentage of clinical isolates for which a zone diameter after a given incubation time could be determined; vME and ME rates >1% and mE with values >5% are shown in bold.

^aCategorical agreement at 6 h and error rates were not calculated due to low readability (average readability 9.2%).

^bAverage values are shown in italics.

for *P. aeruginosa* at 8 h was >90% for β -lactams (piperacillin/tazobactam, ceftazidime, cefepime and imipenem), ciprofloxacin and the aminoglycosides (amikacin, gentamicin and tobramycin). vME and ME rates for *A. baumannii* were low (<1%) at 6 h for the majority of drugs and no ME and vME were observed for *A. baumannii* at 8 h (Table 1).

Discrimination of non-WT and WT populations

Enterococci

WT and non-WT populations were well separated at 6 and 8 h for all examined species/drug combinations including vancomycin-resistant *E. faecium* (n = 62; Table 2 and Figure S1).

Non-fermenters

A. baumannii and *P. aeruginosa* WT and non-WT populations were well separated at early timepoints (>95%) for most species/drug combinations, except for *P. aeruginosa* and the β -lactams, in particular imipenem (Table 2 and Figure S1).

Change of zone diameters and putative ECOFFs during the incubation period

In general, the change of zone diameter was only minor during the incubation period. For 12 of 24 species/drug combinations the ECOFF increased over time (Figure 1). For 11 species/drug combinations, the inhibition zone diameters of the WT population did not

Table 2. Maximal accuracy (in %) for the separation of non-WT and WT populations; cut-offs achieving maximal accuracy are given in parentheses and prevalences were taken from our dataset

Organism	Antibiotic	Maximal accuracy ^a (in %) for the separation of non-WT and WT populations (cut-off) (mm)			
		6 h	8 h	12 h	18 h
<i>E. faecalis</i>	ampicillin	99.2 (8)	99.2 (11)	100 (11)	100 (10)
<i>E. faecalis</i>	gentamicin	96.8 (13)	97.7 (11)	98.5 (8)	99.3 (9)
<i>E. faecalis</i>	norfloxacin	100 (14)	100 (14)	100 (13)	100 (12)
<i>E. faecium</i>	ampicillin	100 (11)	100 (12)	100 (12)	100 (11)
<i>E. faecium</i>	gentamicin	98.9 (11)	99.1 (11)	99.6 (9)	100 (8)
<i>E. faecium</i>	norfloxacin	100 (15)	100 (15)	100 (14)	100 (13)
<i>E. faecium</i>	vancomycin	98 (13)	98.2 (13)	99.1 (11)	100 (11)
<i>P. aeruginosa</i>	piperacillin/tazobactam	NA	96 (16)	96.7 (18)	100 (18)
<i>P. aeruginosa</i>	cefepime	NA	95.5 (19)	98.2 (20)	100 (19)
<i>P. aeruginosa</i>	ceftazidime	NA	95.6 (16)	95.6 (17)	100 (17)
<i>P. aeruginosa</i>	imipenem	NA	86.9 (20)	93.8 (25)	100 (25)
<i>P. aeruginosa</i>	meropenem	NA	95.1 (21)	96.7 (24)	100 (24)
<i>P. aeruginosa</i>	gentamicin	NA	99.5 (14)	99.3 (15)	100 (15)
<i>P. aeruginosa</i>	tobramycin	NA	100 (17)	98.9 (16)	100 (16)
<i>P. aeruginosa</i>	amikacin	NA	98 (15)	99.3 (18)	100 (18)
<i>P. aeruginosa</i>	ciprofloxacin	NA	97.6 (22)	95.3 (25)	100 (25)
<i>A. baumannii</i>	imipenem	98.7 (20)	100 (24)	100 (24)	100 (24)
<i>A. baumannii</i>	meropenem	98.7 (15)	100 (21)	100 (22)	100 (22)
<i>A. baumannii</i>	gentamicin	99.4 (16)	100 (16)	100 (16)	100 (16)
<i>A. baumannii</i>	tobramycin	99.4 (14)	100 (15)	100 (15)	100 (14)
<i>A. baumannii</i>	amikacin	99.4 (17)	100 (18)	100 (18)	100 (18)
<i>A. baumannii</i>	ciprofloxacin	100 (17)	100 (18)	100 (18)	100 (18)
<i>A. baumannii</i>	levofloxacin	99.4 (19)	100 (20)	100 (20)	100 (19)

NA, not applicable.

^aMaximal accuracy as a measure for separation of two populations requires cut-offs. Using a definitory approach, cut-offs for the different incubation period/species/antibiotic combinations were set and the maximum accuracy of the separation was calculated (in %).

change during the incubation period, and for one combination diameters decreased over time (*E. faecium* and gentamicin).

Discussion

We recently demonstrated in a proof-of-principle study the feasibility of rAST.¹⁵ *E. faecalis*, *E. faecium*, *P. aeruginosa* and *A. baumannii* represent a significant part of microorganisms isolated from critically ill patients. We here evaluated whether early reading of disc diffusion can be applied to these pathogens. As reading times 6, 8 and 12 h were chosen in comparison with standard 18 h evaluation.

Readability at early timepoints differed for the species studied: on average, zone diameters of *A. baumannii* and enterococci were readable for >99% and >90% of isolates after 6 h, respectively. *P. aeruginosa* showed an average readability of 68.9% at 8 h and 93.4% at 12 h (Table 1). Extended incubation times related to insufficient growth of *P. aeruginosa* have also been reported in automated antimicrobial susceptibility test devices.²²

For *A. baumannii*, WT and non-WT populations were well separated at 6 and 8 h (Table 2 and Figure S1). When applying the 18 h EUCAST CBPs, ME rates (false-resistant results) of >1% were only seen for ciprofloxacin and tobramycin at 6 h and average vME rates (false-susceptible results) were 0.2% at 6 h and 0% at 8 h.

Therefore, early reading and categorization of *A. baumannii* zone diameters is well feasible at 6 and 8 h applying current 18 h EUCAST CBPs.

For *P. aeruginosa*, we in general observed a good separation of WT and non-WT populations at early 8 h reading (Table 2 and Figure S1). For piperacillin/tazobactam, ceftazidime and imipenem we observed less well separation of WT and non-WT populations, resulting in categorization error rates ranging from 0.5% to 9.7% when applying current 18 h EUCAST CBPs (Table 1). An upcoming task will be the development of species-specific CBPs for early reading timepoints. In addition, rAST of *P. aeruginosa* may need zones of methodological uncertainty that will cover the diameter overlap of WT and non-WT populations and that will serve as buffer zones to prevent categorization errors. Most clinical strains of *P. aeruginosa* display zone diameters that are outside the zone of overlap and can therefore be assigned to the susceptible/WT or the resistant/non-WT population. Ruling out resistance will be possible for a significant number of strains and facilitate proper decision-making for initial antibiotic therapy.

For enterococci, non-WT and WT populations were well separated at early reading times (Table 2 and Figure S1). When current 18 h EUCAST CBPs were applied vME rates of 8.7% and 1.6% for *E. faecalis* and gentamicin and norfloxacin at 6 h were observed. For *E. faecium*, we found vME rates of 6.9% and 2.5% for

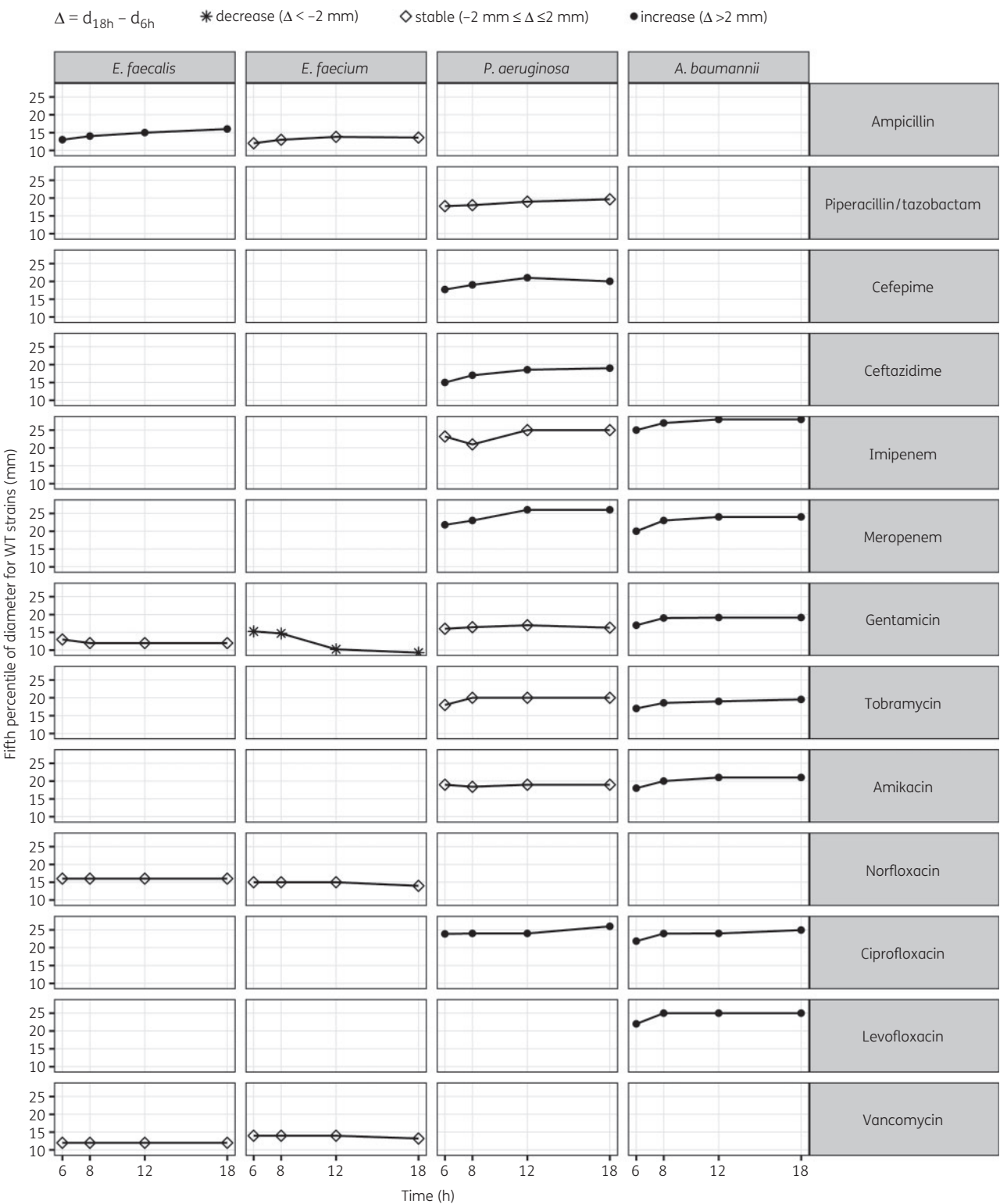


Figure 1. Graphs depict the fifth percentile of the WT population. This value was used as surrogate for the ECOFF as it indicates the lower end of the WT population. Increasing values (lines with filled circles) or decreasing values (lines with asterisks) depict absolute diameter changes of >2 mm between 6 and 18 h. Stable values (absolute diameter changes of ≤ 2 mm between 6 and 18 h) are displayed as lines with diamonds.

gentamicin and vancomycin at 6 h, respectively (Table 1). Incubation period-adapted CBPs or zones of methodological uncertainty will improve categorization into WT and non-WT phenotypes. This is of particular relevance for VRE, as 3 of 65 VRE clinical isolates would have been missed when using the 24 h-based CBP. The current EUCAST 24 h vancomycin $\geq S$ / $<R$ CBP is 12 mm. If, e.g. vancomycin diameters of 12–13 mm were considered inconclusive at 6 or 8 h, the rate of vME in *E. faecium* would drop from 2.5% to 0%.

In conclusion, this study demonstrates that rAST, with reading at 6 h, is possible for *A. baumannii*, *E. faecalis* and *E. faecium*. Only few CBP changes and/or buffer zones will be needed to avoid ME and vME. In addition, rAST with reading after 8 h is possible for *P. aeruginosa*, but it will require buffer zones, covering the overlap of WT and non-WT populations, to prevent erroneous categorizations, in particular for the β -lactams. As a further limitation, this single-centre study cannot issue generally applicable CBPs for rAST, as aggregated population data from various epidemiological settings are recommended for proper CBP determination. We conclude that rAST at 6 or 8 h is feasible for important drug classes for *A. baumannii*, *E. faecalis* and *E. faecium*, and with some limitations for *P. aeruginosa*.

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Transparency declarations

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Supplementary data

Figure S1 and Table S1 are available as [Supplementary data](#) at JAC Online.

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